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Naloxone-Induced Hypoalgesia: Lack of Involvement of the GABA–Benzodiazepine Receptor Complex

JOSEPH ROCHFORD[1] AND JANE STEWART

Center for Studies in Behavioral Neurobiology, Department of Psychology, Concordia University, Montreal, Quebec, Canada H3G 1M8

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ROCHFORD, J. AND J. STEWART. *Naloxone-induced hypoalgesia: Lack of involvement of the GABA-benzodiazepine receptor complex.* PHARMACOL BIOCHEM BEHAV **43**(2) 321–328, 1992.—Previous evidence has demonstrated that repeated daily administration of the opiate receptor antagonist naloxone prior to assessment of pain sensitivity provokes the development of a nonopioid form of hypoalgesia. The present experiments assessed whether the GABA-benzodiazepine receptor complex may be involved in the mediation of this effect. Male Wistar rats were administered 10 mg/kg naloxone prior to hot-plate tests (48.5°C) for pain sensitivity for 8 consecutive days. Control animals were administered saline prior to, and naloxone 2–4 h after, assessment of pain reactivity. Beginning on the fourth or fifth day of this regimen, animals tested under the influence of naloxone displayed longer paw-lick latencies than controls. Preadministration of the $GABA_A$ agonist muscimol (1.0–5.0 mg/kg) and $GABA_A$ antagonist bicuculline (0.25–1.0 mg/kg) failed to affect paw-lick latencies in naloxone-tested and control rats. The $GABA_B$ receptor agonist baclofen (1.0–5.0 mg/kg) and the benzodiazepine receptor agonist diazepam (1.0–5.0 mg/kg) both elevated paw-lick latencies to the same degree in both groups of animals. These results suggest that the GABA-benzodiazepine receptor complex is not involved in the mediation of naloxone-induced hypoalgesia.

Naloxone GABA Benzodiazepine Hypoalgesia Rat

THERE is now extensive evidence demonstrating the existence of endogenous pain-inhibitory substrates within the CNS (4,5,54). Evidence suggests that antinociceptive substrates can be classified into two general categories based upon the neurotransmitters that mediate the pain inhibition. First, endogenous hypoalgesia can be mediated by the release of opioid peptides (i.e., enkephalins, endorphins, dynorphins), as evidenced by the facts that the hypoalgesia can be attenuated by opiate receptor antagonists and displays cross-tolerance with morphine-induced hypoalgesia (21,30,31). Some forms of endogenous hypoalgesia are highly resistant to opiate receptor blockade and do not display cross-tolerance with morphine-induced hypoalgesia (6,7,21,30,31). These latter results suggest the existence of a second, nonopioid antinociceptive substrate.

The results of experiments from three independent laboratories have demonstrated that nonopioid hypoalgesia can be elicited by opiate receptor blockade (11,44,55). Rochford and Stewart (44) administered single daily injections of 10 mg/kg naloxone to rats prior to either hot-plate or tail-flick tests for pain sensitivity for 8 days. Beginning on the fourth or fifth day of this regimen, animals tested under the influence of naloxone were hypoalgesic relative to control animals administered naloxone 2–4 h after pain assessment. Because control animals did not display hypoalgesia, the development of naloxone-induced hypoalgesia is dependent upon receptor blockade at the time pain sensitivity is assessed and is not simply a consequence of repeated opiate receptor blockade.

Three lines of evidence suggest that naloxone-induced hypoalgesia is nonopioid in nature. First, the magnitude of the effect increases as the dose of naloxone administered is increased, suggesting that the effect covaries with the extent to which the opioid pain-inhibitory component is blocked (44,55). Second, there is no cross-tolerance between morphine- and naloxone-induced hypoalgesia (11,55). Third, the hypoalgesia provoked by opiate receptor blockade summates with nonopioid-mediated hypoalgesia provoked by exposure to continuous foot-shock or cold water swim stress (28,44).

Although naloxone-induced hypoalgesia has been characterized as nonopioid in nature, the neurotransmitter systems mediating this effect have yet to be fully explicated. We (43) recently reported evidence implicating noradrenergic substrates in the mediation of this effect. Specifically, naloxone-induced hypoalgesia is attenuated by preadministration of the

[1] Requests for reprints should be addressed to Joseph Rochford, Douglas Hospital Research Center, 6875 Boul. LaSalle, Verdun, Quebec, Canada H4H 1R3.

noradrenergic α_2-receptor agonist clonidine and enhanced by the α_2-receptor antagonist yohimbine.

The present experiments were conducted to determine the influence of ligands that influence the GABA-benzodiazepine (BDZ) receptor complex. We hypothesized that the GABA-BDZ receptor complex may be involved in naloxone-induced hypoalgesia for two reasons. First, within the dose range required to induce hypoalgesia (2.5–10 mg/kg) naloxone has been shown to influence GABAergic substrates (10,16,48). Consequently, it is possible that naloxone might induce its hypoalgesic effect, at least in part, through its GABAergic effects. Second, there is evidence that clonidine and yohimbine possess, respectively, anxiolytic and anxiogenic properties (14,25,39,50). This suggested that the effects of these ligands on naloxone-induced hypoalgesia might be attributable to the manner in which they modulate anxiety. Because BDZs are also potent anxiolytics (22), it was postulated that BDZ preadministration might also attenuate naloxone-induced hypoalgesia. In the present experiments, we examined the effects of baclofen, a $GABA_B$ receptor agonist, muscimol, a $GABA_A$ receptor agonist, bicuculline, a $GABA_A$ receptor antagonist, and diazepam, a BDZ receptor agonist.

METHOD

Subjects

Subjects were experimentally naive, male Wistar rats obtained from Charles River Breeding Farms (St. Constant, Quebec). Rats weighed between 275–300 g upon arrival at our laboratory. They were individually housed and provided with free access to both food and water. The colony room was maintained on a 12 L:12 D cycle. All procedures were conducted during the light phase of the cycle.

Apparatus and Drugs

Pain sensitivity was assessed by the hot-plate test. The hot-plate apparatus consisted of a 20 × 20 × 38-cm clear Plexiglas chamber mounted on a 0.6-cm thick, 27 × 31-cm piece of sheet metal. A hinged, wire mesh lid mounted on the top of the chamber prevented animals from escaping. The plate temperature was controlled by immersing the sheet metal in a water bath heated by an Haake (Berlin, Germany) E2 Immersion/Open Bath Circulator. The apparatus was located in a test room illuminated by two 25-W, red light bulbs and maintained at a constant 22°C temperature. Animals were isolated in the test room in separate 30 × 20 × 15-cm wooden boxes, which were lined with Beta-Chip and covered by steel grid tops.

Drugs used were: naloxone HCl (Dupont de Nemours & Co., Wilmington, DE); baclofen HCl, muscimol HBr, and bicuculline (Research Biochemicals, Inc., Natick, MA); and diazepam (Sabex International, Montreal, Quebec). Naloxone, baclofen, and muscimol were dissolved in physiological saline. Diazepam was dissolved in a vehicle of 50% propylene glycol and 50% saline. Bicuculline was dissolved in a few drops of 1 N hydrochloric acid, diluted with distilled water, and then the pH was adjusted to 5.5 by the addition of 1 N NaOH. The injection volume in each case was 1 ml/kg.

Procedure

The protocol followed was identical for each of the four experiments conducted. Upon arrival, animals were weighed and handled for approximately 1 min each day for 5 days prior to the start of the experiment.

The first phase of each experiment constituted the naloxone treatment phase. During the 8 days of this phase, naloxone-treated rats (NAL) (n = 24 per experiment) were administered 10 mg/kg naloxone in the test room. Saline-treated rats (SAL) (n = 24 per experiment) received an equivalent volume of saline. Test room injections were administered SC in the dorsal neck area. Thirty minutes following injection, each animal was tested for pain sensitivity on the hot plate. The latency to lick a hind paw [paw-lick latency (PLL)] was manually recorded to the nearest 0.1 s with a timer (Lafayette Instrument Co., Chicago, IL). The water temperature of the hot-plate bath was 48.5 (±0.2)°C. This low temperature was used because previous work in our laboratory has shown it to be ideal for the manifestation of naloxone-induced hypoalgesia (44). If no response was observed within 90 s, the test was terminated and a PLL of 90 s was recorded. Following the hot-plate test, animals were returned to their home cages where, between 2 and 4 h after the hot-plate test, NAL rats were injected with saline and those in the SAL condition were administered 10 mg/kg naloxone.

The second phase of each experiment was designated the test phase. Rats within the NAL condition were matched on the basis of their mean PLLs from the last 2 days of the naloxone treatment phase and allocated to three groups (n = 8). Rats in the SAL condition were also allocated to groups in this manner. The groups differed with respect to the dose of drug administered in the test phase (see below).

The test phase for each experiment consisted of 2 days. Half the animals within each group were administered an IP injection of drug in the colony room on the first test day and vehicle on the second day. For the other half of the animals, the sequence was reversed. Thus, the sequence of colony room injections within the 2 test days was counterbalanced within groups. The drugs administered in each experiment were: Experiment 1—baclofen (1.0, 2.5, or 5.0 mg/kg); Experiment 2—muscimol (1.0, 2.5, or 5.0 mg/kg); Experiment 3—bicuculline (0.5, 1.0, or 2.0 mg/kg); and Experiment 4—diazepam (1.0, 2.5, and 5.0 mg/kg). In each experiment, the vehicle injected was the solution used to dissolve the drug [i.e., saline for baclofen and muscimol, acidified saline (pH = 5.5) for bicuculline, 50% propylene glycol, 50% saline for diazepam].

Fifteen minutes following colony room injection, animals were transported to the test room where rats in the NAL groups were injected with naloxone and those in the SAL groups received saline. Thirty minutes later, animals were administered a hot-plate test and were then returned to the colony room, where, 2–4 h later, NAL rats were administered saline and SAL rats received naloxone.

Statistical Analysis

Separate analyses of variance (ANOVAs) were conducted on the data from the naloxone treatment and test phases of each experiment. Pairwise comparisons were conducted using F-tests for simple main effects (57, pp.529–532). The level of statistical significance adopted was $p < 0.05$.

RESULTS

Experiment 1: Effects of Baclofen

The results from the naloxone treatment phase of Experiment 1 are displayed in Fig. 1. The split-plot, condition × days ANOVA conducted on the data yielded a significant interaction between these two factors, $F(1, 322) = 4.09$, $p < 0.001$. Simple main effects F-tests revealed that the mean

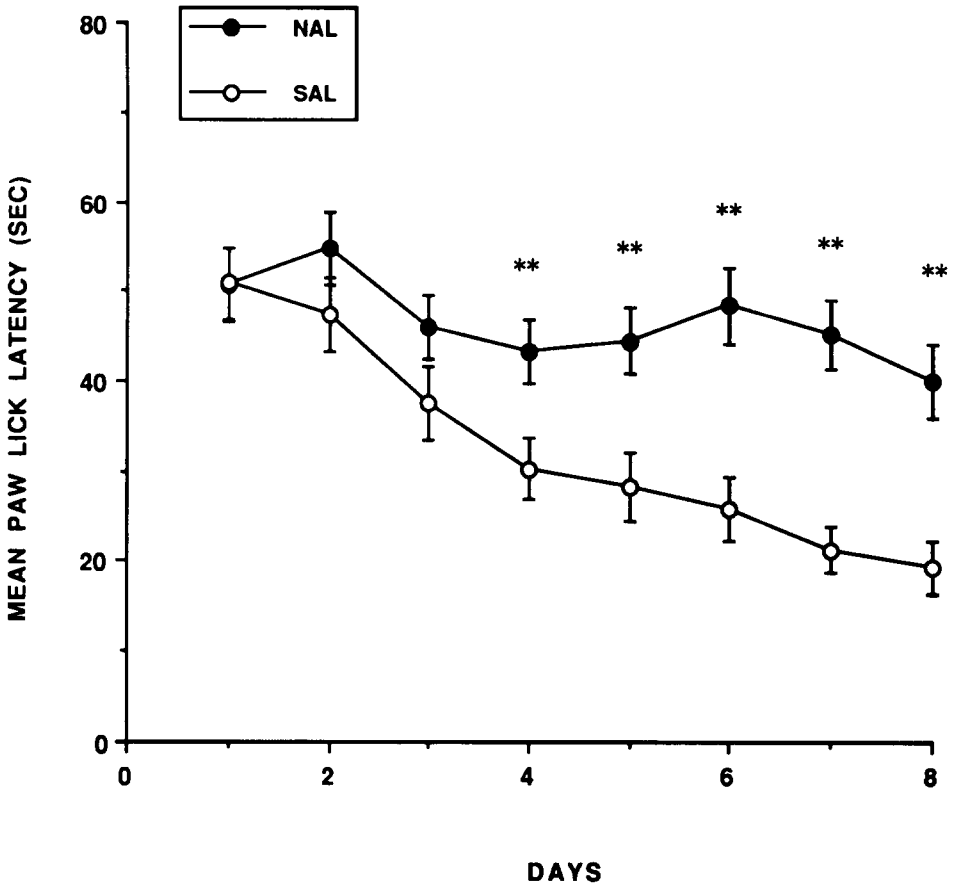

FIG. 1. Mean (±SEM) paw-lick latencies for rats in the NAL and SAL conditions for the 8 days of the naloxone treatment phase of Experiment 1. Asterisks represent significant differences between conditions, $^{**}p < 0.025$.

latencies between conditions did not differ over the first 3 days, $Fs(1, 184) \leq 2.51$, $ps > 0.05$, whereas from days 4–8 rats in the NAL condition displayed significantly longer PLLs than those in the SAL condition, $Fs(1, 184) \geq 6.01$, $ps < 0.025$.

Preliminary analysis of the data from the test phase of this and subsequent experiments did not yield significant main effects for counterbalancing of the home cage injection sequence (i.e., drug–vehicle vs. vehicle–drug). Nor did this variable interact significantly with any other variable. Consequently, the data from the test phases of all four experiments were collapsed over this factor.

The results from the test phase of Experiment 1 are displayed in Fig. 2. The data were analyzed by a split plot, condition (NAL vs. SAL) × baclofen dose × home cage injection (baclofen vs. vehicle) ANOVA. This yielded a significant main effect for condition, $F(1, 42) = 9.65$, $p < 0.005$, as well as a significant baclofen dose × home cage injection interaction, $F(1, 42) = 12.61$, $p < 0.001$. The condition main effect reflects the fact that rats in the NAL condition continued to display longer latencies in the test phase than those in the SAL condition. The dose × injection interaction is attributable to the fact that preadministration of 2.5 and 5.0 mg/kg baclofen elevated latencies in comparison to vehicle preadministration, $Fs(1, 42) \geq 5.48$, $ps < 0.025$, whereas administration of 1.0 mg/kg was without effect, $F(1, 42) = 0.88$, $p > 0.05$. Baclofen did not differentially alter latencies in the NAL and SAL conditions as the condition × home cage injection, $F(1, 42) = 1.99$, and the condition × dose × home cage injection, $F(1, 42) = 0.41$, interactions were not significant, $ps > 0.05$.

Experiment 2: Effects of Muscimol

The ANOVA conducted on the results from the naloxone-treatment phase of Experiment 2 (data not shown) revealed a significant condition × days interaction, $F(7, 322) = 3.90$, $p < 0.001$. F-tests for simple main effects revealed that over the first 3 days the mean PLLs from animals in the NAL condition (range: 45.54–60.70) were no different from those in the SAL condition (range: 38.94–51.61), $Fs(1, 213) < 1.0$, $ps > 0.05$. The mean PLLs in naloxone-treated animals over days 4–8 (range: 44.85–53.36) were significantly longer than those in the SAL condition (range: 34.65–18.17), $Fs(1, 213) \geq 11.73$, $ps < 0.001$.

Figure 3 displays the test phase results. ANOVA revealed a significant main effects for condition, $F(1, 42) = 54.61$, $p < 0.001$, reflecting the fact that naloxone-tested animals displayed longer PLLs than rats tested under saline. Muscimol had no effect on PLLs, as all other main effects and interactions were not significant ($ps > 0.05$).

Experiment 3: Effects of Bicuculline

The ANOVA conducted on the results from the naloxone treatment phase of Experiment 3 (data not shown) revealed a

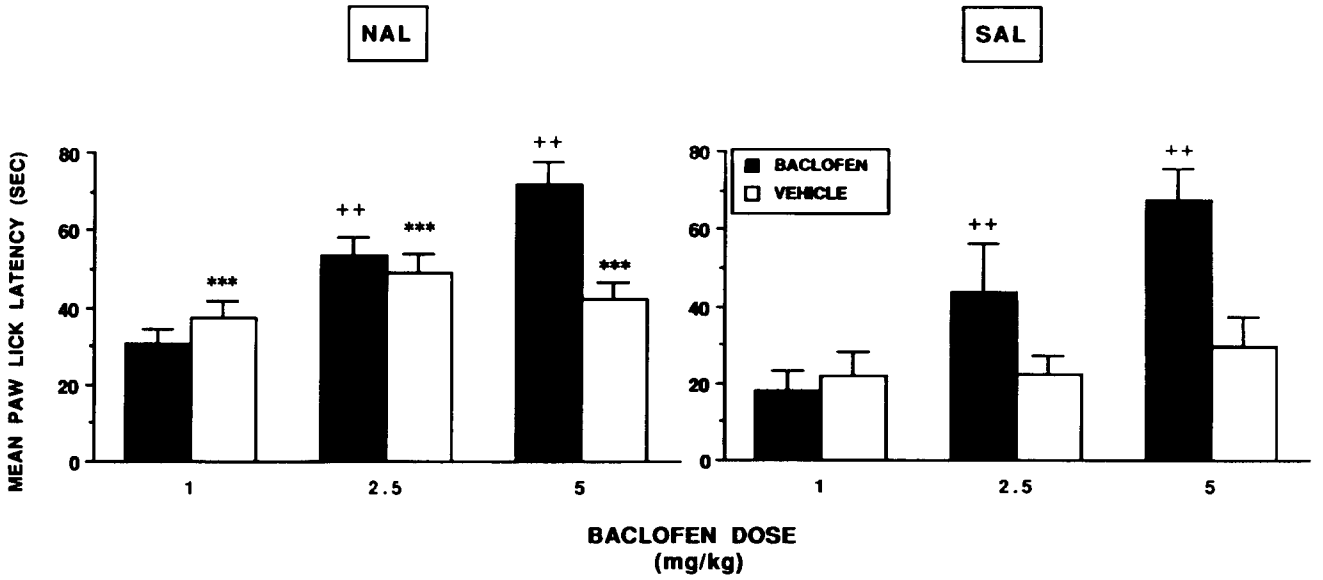

FIG. 2. Mean (±SEM) paw-lick latencies for the test phase of Experiment 1. The left panel displays the results from the three groups within the NAL condition and the right panel those from the SAL condition. The abscissa indicates the dose of baclofen administered to each group. Asterisks reflect the fact that the mean latencies for the three NAL groups were significantly longer than the latencies for the respective groups in the SAL condition, $***p < 0.001$. Plus signs indicate that the combined mean for animals in the NAL and SAL conditions (i.e., the mean formed by collapsing over condition within each dose of baclofen) was significantly longer following baclofen administration than following vehicle, $^{++}p < 0.025$. See text for further details.

significant condition × days interaction, $F(7, 322) = 6.21$, $p < 0.001$. Over the first 4 days, the mean PLLs from animals in the NAL condition (range: 44.50–56.79) were no different from those in the SAL condition (range: 36.49–51.58), $Fs(1, 213) < 1.0$, $ps > 0.05$, but naloxone-tested animals did display significantly longer mean latencies (range: 44.08–48.25) than saline-tested rats (range: 19.99–28.35) over days 5–8, $Fs(1, 165) \geq 18.42$, $ps < 0.001$.

Figure 4 displays the results of the test phase of this experiment. Inspection of Fig. 4 reveals that bicuculline exerted little influence on the PLLs of animals in either the NAL or SAL conditions. This was confirmed statistically as only the main effect for condition (revealing that NAL condition animals displayed longer PLLs than SAL condition animals) was significant, $F(1, 42) = 24.03$, $p < 0.001$.

Experiment 4: Effects of Diazepam

The ANOVA for the naloxone treatment phase yielded a significant condition × days interaction, $F(7, 322) = 6.85$,

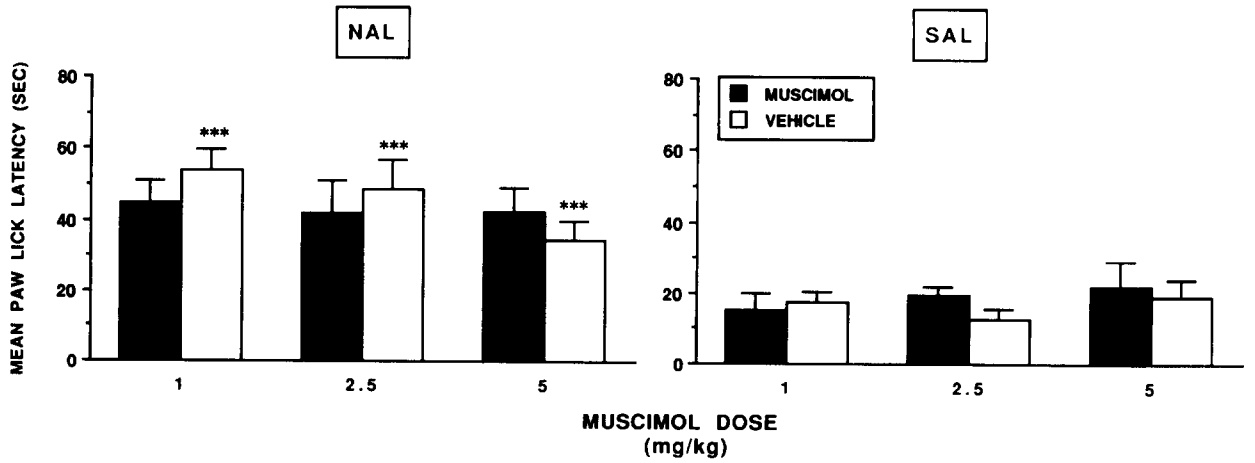

FIG. 3. Mean (±SEM) paw-lick latencies for the test phase of Experiment 2. The left panel displays the results from the three groups within the NAL condition and the right panel those from the SAL condition. The abscissa indicates the dose of muscimol administered to each group. Asterisks reflect the fact that the mean latencies for the three NAL groups were significantly longer than the latencies for the respective groups in the SAL condition, $***p < 0.001$.

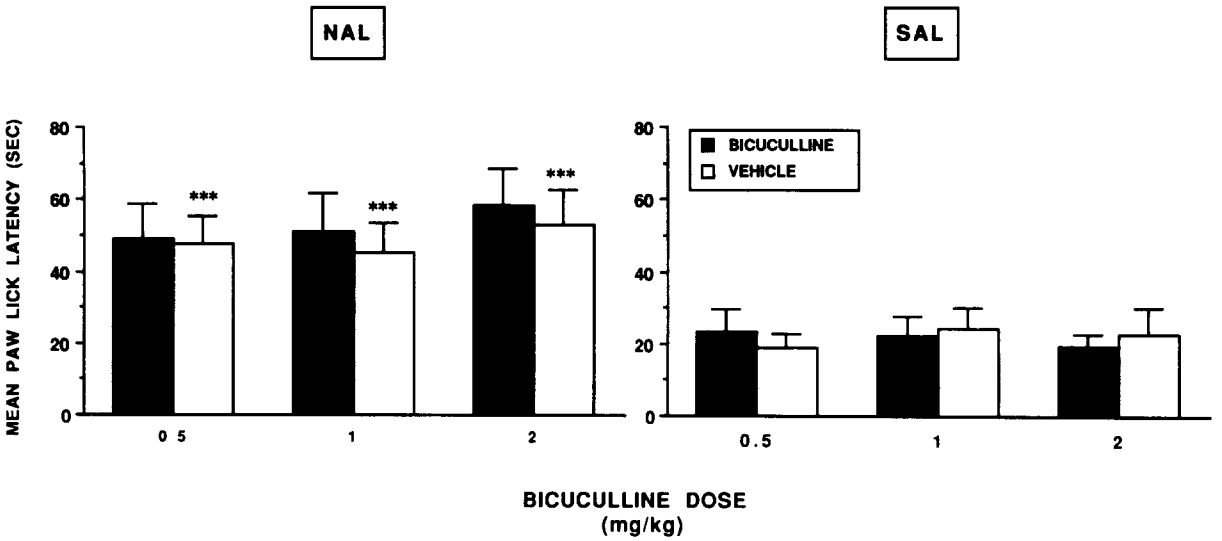

FIG. 4. Mean (±SEM) paw-lick latencies for the test phase of Experiment 3. The left panel displays the results from the three groups within the NAL condition and the right panel those from the SAL condition. The abscissa indicates the dose of bicuculline administered to each group. Asterisks reflect the fact that the mean latencies for the three NAL groups were significantly longer than the latencies for the respective groups in the SAL condition, $^{***}p < 0.001$.

$p < 0.001$. The mean PLLs between conditions (range: 62.35–51.32 for the NAL condition, 55.72–64.34 for the SAL condition) did not differ significantly for the first 3 days, Fs(1, 224) ≤ 1.41, ps > 0.05. Naloxone-tested animals did display significantly longer mean latencies (range: 47.41–53.26) than saline-tested rats (range: 23.05–33.49) over days 4–8, Fs(1, 165) ≥ 18.42, ps < 0.001.

Figure 5 displays the results from the test phase of Experiment 4. ANOVA yielded significant main effects for condition, $F(1, 42) = 41.59$, $p < 0.001$, and home cage injection, $F(1, 42) = 26.57$, $p < 0.001$. These results indicate that NAL rats displayed significantly longer latencies than SAL rats and that diazepam elevated latencies in all animals. Diazepam's augmentation of PLLs was not differential between the two conditions, as the condition × home cage injection and the condition × dose × home cage injection interactions were not significant, Fs < 1.0, ps > 0.05.

DISCUSSION

Consistent with previous reports (11,44,55), the results of the present experiments demonstrate that daily administration of the opiate receptor blocker naloxone prior to hot-plate tests for pain sensitivity results in the development of hypoalgesia.

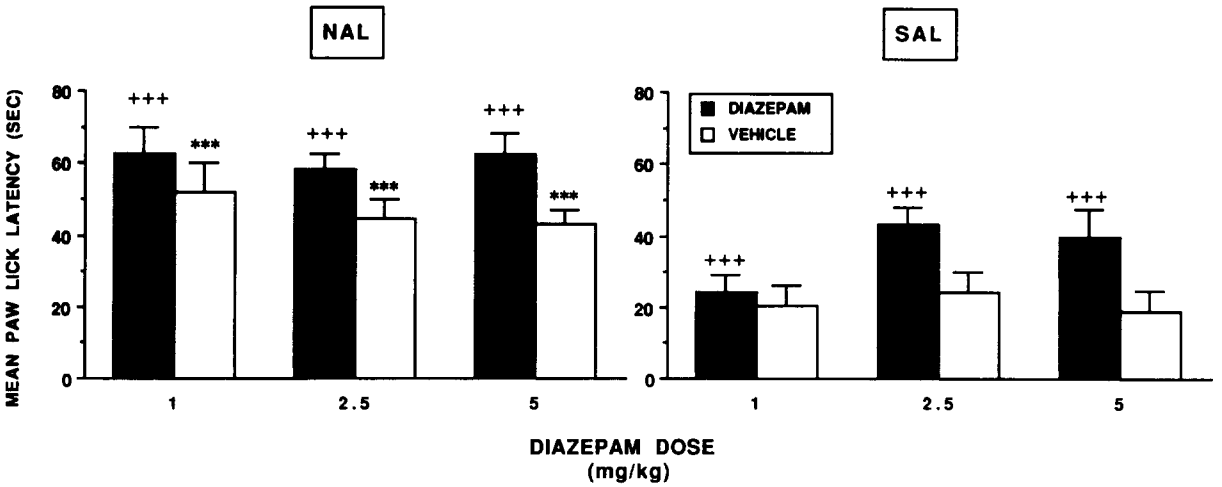

FIG. 5. Mean (±SEM) paw-lick latencies for the test phase of Experiment 4. The left panel displays the results from the three groups within the NAL condition and the right panel those from the SAL condition. The abscissa indicates the dose of diazepam administered to each group. Asterisks reflect the fact that the mean latencies for the three NAL groups were significantly longer than the latencies for the respective groups in the SAL condition, $^{***}p < 0.001$. Plus signs indicate that the combined mean for animals in the NAL and SAL conditions (i.e., the mean formed by collapsing over condition within each dose of diazepam) was significantly longer following diazepam administration than following vehicle, $^{+++}p < 0.001$. See text for further details.

In each experiment, animals in the NAL condition began to display longer PLLs beginning on the fourth or fifth day of treatment. This hypoalgesic effect of naloxone persisted throughout the remainder of the naloxone treatment phase, as well as throughout the test phase.

It is worth noting that naloxone-induced hypoalgesia arises from a decline in the latencies of animals in the SAL condition; the latencies of naloxone-tested animals remained relatively stable over the course of testing. It has been previously suggested (44) that naloxone-induced hypoalgesia is attributable, at least in part, to the fact that naloxone prevents the attenuation of novelty stress-induced hypoalgesia, that is, during the first few hot-plate tests the latencies among animals in both the NAL and SAL conditions are high because of the stress provoked by the novelty of the hot-plate apparatus [cf. (1,3,20,49)]. Over subsequent tests, novelty-induced hypoalgesia diminishes in animals in the SAL condition. Naloxone administration retards this process; consequently, animals in the NAL condition display longer PLLs during the latter part of the naloxone treatment phase and test phase.

We had hypothesized that naloxone-induced hypoalgesia may be at least partly mediated through naloxone's action on the GABA–BDZ receptor complex (10,16,48). The results from the present experiments did not confirm this hypothesis. Of the four ligands examined, bicuculline and muscimol failed to alter pain reactivity. The failure of these ligands is not likely attributable to insufficient dose because previous work has demonstrated that these ligands provoke centrally mediated effects within the dose ranges employed (9,12,15,24). Baclofen and diazepam elevated latencies in both NAL and SAL animals, and the magnitude of this elevation was similar in both groups. These results suggest that the effects provoked by these ligands occurred through an action on a substrate orthogonal to the substrate activated by naloxone administration [cf. (27)], that is, the hypoalgesia provoked by naloxone is mediated through a substrate that does not involve the GABA–BDZ complex. The augmentation provided by baclofen and diazepam reflects the activation of a second, GABA–BDZ-dependent substrate.

The conclusion that GABA–BDZ substrates do not mediate naloxone-induced hypoalgesia may be criticized on the basis of our failure to assess the influence of ligands that directly bind to the chloride channel of the $GABA_A$–BDZ–chloride channel complex, such as picrotoxin and *t*-butylbicyclophosphorothionate (TBPS) (37,38,41). However, it is now apparent that ligands that bind to either the GABA or BDZ site of the complex allosterically modulate the chloride channel (33,35,36,40,51). Thus, the finding that a $GABA_A$ agonist (muscimol), a $GABA_A$ antagonist (bicuculline), and a BDZ agonist (diazepam) were either without effect or provoked effects not specific to naloxone-treated animals suggests that the chloride channel of the complex does not modulate naloxone-induced hypoalgesia.

There is some controversy in the literature concerning whether the changes in behavioral indices of pain sensitivity induced by GABA and BDZ receptor ligands reflect true antinociception or are attributable to sedative effects (8,23,47,53). Our data do not address this issue, and we believe it is not directly relevant to the principle focus and conclusion of the present experiments. The experiments were conducted to determine what role, if any, the GABA–BDZ complex plays in the mediation of naloxone-induced hypoalgesia. Our conclusion is that because baclofen and diazepam influenced PLLs to the same extent in naloxone- and saline-tested animals the mediational role of the GABA–BDZ complex is negligible. This conclusion is valid whether the alterations provided by these ligands are attributed to their putative antinociceptive effects or their sedative effects.

Naloxone-induced hypoalgesia is attenuated by preadministration of the noradrenergic α_2-receptor agonist clonidine and enhanced by the α_2-receptor antagonist yohimbine (43). Because these ligands have been reported to possess, respectively, anxiolytic and anxiogenic properties (14,25,39,50), this raised the possibility that the effects of these ligands on naloxone-induced hypoalgesia may have been mediated through their actions on neuroanatomic substrates mediating anxiety. The results from the test phase of Experiment 4 did not provide any support for this hypothesis. Diazepam, a potent anxiolytic (22), did not mimic clonidine's inhibitory effect on naloxone-induced hypoalgesia, suggesting that naloxone-induced hypoalgesia is not mediated through its effects on substrates involved in anxiety. This result stands in contrast to those from studies that have demonstrated that other forms of hypoalgesia can be attenuated by BDZ preadministration (17–19,31,46,56). However, a closer examination of these studies reveals that each of these forms of hypoalgesia involve opioid antinociceptive substrates. Most studies that have focused on the effects of BDZ administration on nonopioid forms of hypoalgesia have found them to be resistant to BDZ administration [(13,30,34), but see (8,45)]. Consequently, it is likely that diazepam did not attenuate naloxone-induced hypoalgesia because this effect is nonopioid in nature (see the introductory section).

Taken in combination with the result that clonidine is able to inhibit naloxone-induced hypoalgesia, the failure of diazepam to do so raises an apparent paradox. Clonidine's attenuating action on naloxone-induced hypoalgesia was interpreted (43) as reflecting the inhibition of noradrenergic neurotransmission provoked by this ligand through its actions on inhibitory α_2-noradrenergic receptors located presynaptically on noradrenergic neurons (2,26,52). The source of the paradox stems from the observation that diazepam also inhibits noradrenergic neurotransmission (42).

This paradox may be resolved by acknowledging the existence of multiple central noradrenergic pathways, only some of which are sensitive to BDZ administration (32). Those studies demonstrating BDZ inhibition of noradrenergic function have focused primarily on the A6 locus coeruleus (LC) region. This region has been suggested to be vital in anxiogenesis, and the inhibition of LC neurons provoked by BDZ administration is considered one of the primary mechanisms through which these ligands provide anxiolysis (14). Whereas the LC has also been implicated in the modulation of pain sensitivity, it is equally true that other anatomically distinct noradrenergic pathways, such as those arising from the ventromedial medulla, contribute to antinociception [for review, see (5)]. Moreover, to the best of our knowledge, these latter pathways do not respond to BDZ administration. It may be, therefore, that the reversal of naloxone-induced hypoalgesia provoked by clonidine is mediated through the inhibition of nonadrenergic pathways other than the LC.

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